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Running title: hepatocellular adenoma classification

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Abstract

Hepatocellular adenomas are benign tumors that can be difficult to diagnose. To refine their classification, we performed a comprehensive analysis of their genetic, pathological and clinical features. A multicentric series of 96 liver tumors with a firm or possible diagnosis of hepatocellular adenoma was reviewed by liver pathologists. In all cases, the genes coding for hepatocyte nuclear factor 1α (HNF1α) and ß-catenin were sequenced. No tumors were mutated in both HNF1α and ß-catenin enabling tumors to be classified into 3 groups, according to genotype. Tumors with HNF1α mutations formed the most important group of adenomas (44 cases). They were phenotypically characterized by marked steatosis ($p<10^{-4}$), lack of cytological abnormalities ($p<10^{-6}$) and inflammatory infiltrates ($p<10^{-4}$). In contrast, the group of tumors defined by ß-catenin activation included 13 lesions with frequent cytological abnormalities and pseudo-glandular formation ($p<10^{-5}$). The third group of tumors without mutation, was divided in two subgroups based on the presence of inflamatory infiltrates. The subgroup of tumors consisting in 17 inflammatory lesions, resembled telangiectatic focal nodular hyperplasias, with frequent cytological abnormalities ($p=10^{-3}$), ductular reaction ($p<10^{-2}$) and dystrophic vessels ($p=0.02$). In this classification, hepatocellular carcinoma associated with adenoma or borderline lesions between carcinoma and adenoma is found in 46% of the ß-catenin mutated tumors whereas they are never observed in inflammatory lesions and are rarely found in HNF1α mutated tumors ($p=0.004$). The molecular and pathological classification of hepatocellular adenomas permits the identification of strong genotype-phenotype correlations and suggests that adenomas with ß-catenin activation have a higher risk of malignant transformation.

Introduction

Hepatocellular adenomas (HA) are rare benign liver tumors, most frequently occurring in women using oral contraception(1). HA are single or more rarely multiple nodules; the presence of more than ten nodules in the liver indicates a specific nosological entity: liver adenomatosis(2). In 1978, Foster and collaborators described another clinical entity, which was the first case of familial adenomatosis associated with diabetes(3), later confirmed by others(4-7). Some small HA may regress after withdrawal of oral contraceptives; however, they usually remain stable, or increase in size. They may bleed, or rarely, undergo malignant transformation(8, 9). Given the unpredictable evolution of these lesions, they are generally surgically removed. Moreover, it may be difficult for pathologists to differentiate HA from well-differentiated hepatocellular carcinoma (HCC), or sometimes from regenerative lesions such as focal nodular hyperplasia (FNH) and particularly from telangiectatic focal nodular hyperplasia(10) (TFNH), recently identified as monoclonal lesions(11, 12) subject to frequent bleeding(11, 12).

Recently, genetic alterations have been identified in HA. Mutations of the *TCF1* gene inactivating the Hepatocyte Nuclear Factor 1α (HNF1α) transcription factor, were identified in half of the HA cases(5) and most frequently both allelic mutations were somatic. Patients with an inherited mutation in one allele of HNF1α may develop maturity onset diabetes of the young type 3(13) (MODY3, OMIM#600496) and familial liver adenomatosis, when the second allele is inactivated in hepatocytes by somatic mutation or chromosome deletion(5-7) (OMIM#142330). These results showed that the HNF1α transcription factor gene fulfils the genetic characteristics of a tumor suppressor gene and is a factor for genetic predisposition to familial hepatocellular tumors(14). Furthermore, HNF1α mutations occur specifically in adenomas among the benign hepatocellular tumors since no mutations were identified either in typical FNH or TFNH

cases(11, 12), in contrast to rare mutations observed in endometrial, colon and renal carcinomas(15-17). Recently, activation of the ß-catenin pathway was also found in HA(18-20). On the other hand, activating mutations of ß-catenin are found in 20 to 34% of hepatocellular carcinomas(21-24) suggesting that ß-catenin is the most frequently activated oncogene in HCC. Furthermore, this pathway plays a key role in liver physiological phenomena, such as lineage specification, differentiation, stem cell renewal, epithelial-mesenchymal transition, proliferation and cell adhesion(25, 26).

The aim of the present study was to characterize HNF1α and ß-catenin mutations in a large series of HA and to relate the molecular findings to the histopathology, in order to clarify the classification of these tumors into homogeneous subgroups that might have different evolutive potential, especially with regard to malignant transformation.

Patients and methods

1 Liver samples and clinical data

A group of 13 French university hospitals has participated in this retrospective study and 96 patients were recruited between 1992 and 2004. Criteria of inclusion in the study were a definite (87 cases) or possible (9 cases) diagnosis of adenoma and an adequate sampling of frozen and fixed liver tissues following hepatectomy (95 cases) or transplantation (1 case). No case previously diagnosed as typical FNH or typical HCC, without associated adenoma, were included in this study. Among the 96 included cases, 18 were previously described(5, 7). For each case, a representative part of the principal nodule, as well as of the non tumoral liver, was immediately frozen in liquid nitrogen and stored at – 80C° until used for molecular studies. When multiple nodules were present, one representative lesion was selected and the same lesion was analyzed by molecular biology and reviewed morphologically. This pathological/genotypic correlation was limited, for technical reasons, to an area of the nodule which was not necrotic or hemorrhagic. In the vast majority of the cases, lesions were macroscopically homogeneous; in five cases in which adenoma and HCC coexist, HCC lesions were also genetically and morphologically analyzed but only adenomas lesions were included in the statistical analysis. Clinical data for oral contraception, individual and familial history, circumstances of diagnosis and the associated diseases were systematically collected. All patients were recruited in accordance with French law and institutional ethical guidelines. The overall design of the study was approved by the ethical committee of hospital Saint-Louis, Paris France.

2 Pathological reviewing

Paraffin tissue sections were stained with hematein-eosin, as well as in most of the cases, with Masson’s trichrome, reticulin and Perls. Additional immunostaining were also performed in some

cases with cytokeratins 7 and 19, α smooth muscle actin, and CD34, in order to better visualize, if necessary, biliary structures, arteries and capillarisation of sinusoids, respectively. A group of 10 liver pathologists (PBS, MF, ESZ, CG, VP, JTVN, J-YS, EL, SM and DW) reanalyzed all cases in 4 collective reviewing sessions and among them, 5 pathologists participated in all sessions to ensure uniform evaluation. For each case a set of 20 variables were systematically recorded, including the macroscopic characteristics of the tumors (size, number, presence of hemorrhage, necrosis), and microscopic features such as the presence of a fibrous capsule, sharp outlines, sinusoidal dilatation, dystrophic vessels included in a connective tissue matrix, multiple vessels (dispersed in the tumor and visible at x 4 magnification), presence of fibrous bands, steatosis (graded as mentioned below), and ductular reaction. In addition, inflammatory infiltrate (either focal or diffuse, visible at 10X magnification) as well as cytological abnormalities (large and irregular nuclei, high nucleo/cytoplasmic ratio, Mallory bodies) and pseudo-glandular formations were also noted. The non-tumor tissue was evaluated for fibrosis, according to the METAVIR score(27), for sinusoidal dilatation and for peliosis. In both tumor and non-tumor tissues, steatosis was evaluated as either absent, or involving less than 1/3 of the hepatocytes, or of 1/3 to 2/3 of the hepatocytes, or of more than 2/3 of the hepatocytes. At the end of every case review, each pathologist made a diagnosis, which was considered definitive if at least 80% of the pathologists agreed and 87% of the initial diagnoses were confirmed by these reviews. The pathological diagnosis of adenoma, HCC, TFNH and FNH was made according to classical criteria(28).

Briefly, typical adenoma corresponded to a proliferation of benign hepatocytes, intermingled with numerous thin-walled vessels, without portal tracts. In typical, solid FNH, pseudo-cirrhotic nodules made of (sub)normal hepatocytes were separated by more or less thick fibrous bands,

mixed with malformed vascular, especially arterial structures, inflammatory cells mainly lymphocytes, and ductular reaction. TFNH corresponded to hepatocellular proliferation without nodular organization but with portal tract-like structures containing thick arterial sections, mixed with inflammatory cells and a more or less obvious ductular reaction. Finally, the diagnosis of well-differentiated HCC was made in case of architectural abnormalities (such as arrangement in more than 3 cell-thick plates, numerous acinar structures), often associated with cytological anomalies. When a clear differential diagnosis was not possible, lesions were considered borderline with two possible diagnoses.

3- Mutation screening

In all tumors, we sequenced the HNF1α and ß-catenin genes using direct sequencing of the exons after amplification of genomic DNA to identify mutations, as previously described(5, 24). In addition, to detect any large ß-catenin deletions we performed a PCR amplification using a 5' located exon2 (GGGTATTTGAAGTATACCATAC) and a 3' located exon 4 (TGGTCCTCGTCATTTAGCAG) primers on DNA templates. In samples that showed over-expression of the ß-catenin target genes (GLUL and GPR49) but without the exon3 ß-catenin activating mutation, we sequenced all of the Axin1 and ß-catenin coding exons using direct sequencing on DNA or RT-PCR amplified templates, respectively (all detailed primers and protocols are available upon request). All mutations were confirmed by sequencing DNA from two independent PCR amplifications of DNA from tumor and corresponding non-tumor tissues.

4- Quantitative RT-PCR

Activation of ß-catenin results in transcriptional activation of targeted genes and the overexpression of GS and GPR49, coding for glutamine synthetase and for an orphan nuclear receptor, respectively, are reliable markers of the ß-catenin activation in HCC (29, 30). In 68

tumors and 8 non-tumor tissues, the quality of RNA extracted using the RNeasy kit (Qiagen) enabled us to perform quantitative RT-PCR experiments as previously described(15). We used sequence detection reagents primers and probes specific for *GS* (glutamine synthetase), *GPR49, 18S* (ribosomal RNA) developed by Applied Biosystems. The relative amount of mRNA in samples, was determined using the $2^{-\Delta\Delta CT}$ method(31). The values obtained were expressed as the n-fold ratio of the gene expression in a tested sample compared to the mean of non-tumor tissues. PCR efficiency was measured using LinRegPCR software(32). All gene assays demonstrated a PCR efficiency superior to 90%.

5- Statistical analysis

Statistical analysis was carried out using Stata 8.0 software (Stata Corp, College Station, TX). Qualitative and categorized quantitative variables were compared to each other in contingency tables using a chi-square statistic or Fisher's exact test. For quantitative variables, data were expressed as mean and its standard error. The differences between quantitative variables were evaluated with a t-test or ANOVA when the variances were similar, or with the Kruskall-Wallis test when the data was heteroskedastic. All reported P values were two-tailed; a P value of less than 0.05 was considered to indicate statistical significance.

Results

1- Clinical and pathological features of the patients.

Among the 96 patients included in the series for a presumed diagnosis of HA, the sex ratio (F:M) was 8:1 and the mean age was 37 years (range 14-78). Among the 83 females, 72% had taken oral contraception for longer than 2 years (mean duration=12 years, excluding 9 cases with missing data). One case was diagnosed during pregnancy and 4 patients presented an associated endometriosis. The presenting symptoms were acute abdominal pain in 28% of the cases, abdominal pain of more than 1 month duration in 26% of the cases, and the remaining cases were discovered on routine check-up or investigation for unrelated disorders. We noted several associated rare pathologies in individual cases, including familial adenomatous polyposis, meningioma, Fanconi anemia (treated by androgen therapy), primary hyperoxaluria type 1, renal dysplasia, Crohn's disease and a portal cavernoma treated by a mesenterico-caval anastomosis. Other associated diseases were: a polycystic kidney disease and a glycogen storage disease type Ia in 2 cases, respectively. Finally an HCV infection combined with alcohol abuse was observed in one case and three independent patients had first degree relatives with liver adenomatosis. Adenomatosis was defined by the presence of 10 or more adenoma nodules in the liver (18 cases), whereas we classified as "multiple adenomas" 27 cases where 2 to 6 nodules were found (no patients presented 7 to 9 nodules) in the liver.

The pathological reviewing led to a final diagnosis with a consensus of the committee in 77 cases: 68 HA, 5 HA with areas of HCC, 3 TFNH and 1 HCC. The remaining 18 cases were classified as borderline: 6 HA/HCC, 6 HA/FNH, and 6 HA/TFNH. Finally, in one case no diagnosis was retained and remained unclassifiable. The corresponding non-tumor liver tissues was normal in 60 cases. In the remaining 36 cases the pathological analysis showed a F2 (2

cases) and F3 (2 cases) fibrosis, a moderate sinusoidal dilatation (5 cases), a steatosis (23 cases), an iron overload (2 cases) and polycystic lesions in 2 cases.

2- Molecular analyses

a- Mutation of HNF1α in adenomas and correlations with phenotype

We identified HNF1α mutations in 44 of the 96 tumors screened (46%). In each case two HNF1α mutations were identified in tumors. Both mutations were of somatic origin in 37 cases (84%), whereas in the remaining 7 patients one mutation was germline and the other was somatic (Figure 1). Mutations were non-sense, frameshifts or splicing alterations in half of the cases (Figure 1 upper panel), gene deletion (LOH) in 15% of the cases and amino-acid substitution in 35% of the cases (Figure 1 lower panel). All but 4 of the mutations leading to amino acid substitution were restricted to the POUH homeodomain and altered an amino-acid conserved through different species (Figure 1 and table 1). Two hotspots of mutations were found at codon 291 and 206. The codon 291 substitution located in a polyC-8 tract is the most frequent mutation found in MODY3 patients, whereas the codon 206 mutation, which was not contained in a repeated sequence motif, was specific to adenomas(33).

Lesions containing HNF1α mutations were mainly adenomas with a firm diagnosis (37 out of the 44 cases; 84%). The other tumors were diagnosed as borderline lesion between adenoma and HCC (2 cases), or between adenoma and FNH (4 cases); in 1 additional case, part of the adenoma was associated with an HCC (Table 2). So mutations in this gene seem to lead the most usual form of adenoma. Analysis of the pathological and clinical data revealed that HNF1α mutations were observed in a homogeneous group of tumors closely associated with marked steatosis ($p<10^{-4}$), and no cytological abnormalities ($p=10^{-5}$, Table 2 and Figure 2) or inflammatory

infiltrate ($p<10^{-4}$). Germline HNF1α mutations defined a specific subgroup of patients who were younger (mean age= 23 vs 40 years old; p=0.0001, T-test), with known familial liver adenomatosis in three of the cases (p=0.003 Fisher). Women with germline HNF1α mutations were less likely to have used oral contraception when compared to women presenting biallelic HNF1α somatic mutations (p=0.002, Fisher). Patients with germline mutations in HNF1α developed larger tumors than patients with biallelic somatic mutations (mean size 142 mm vs 69 mm, p= 0.02 Kruskall Wallis) and tumors were more numerous, with 4 patients presenting more than 10 nodules, larger than 1 cm (p=0.04, Fisher). Furthermore in 5 out of the 7 patients presenting germline mutation, numerous infracentimetric steatotic adenomas were detected in the liver at distance from the principal nodule. The other characteristics did not differ significantly in relation to the germline or somatic origin of the mutation.

b- ß-catenin mutations and tumors over-expressing ß-catenin target genes and correlations with phenotype

We found ß-catenin gene alterations in 12 cases (Table 3). In 6 of these cases we found a large deletion including the exon 3 and frequently most of exon 4. In five cases we found an amino-acid substitution altering a site of phosphorylation by GSK3. In the last case, an amino-acid substitution was observed at lysine 335, an amino-acid which binds E-cadherin and TCF3 when phosphorylated(34). In ß-catenin mutated tumors, two ß-catenin target genes, GS and GPR49, were over-expressed 42-fold (ranging from 9 to 87) and 35-fold (ranging from 8 to 57) when compared with non-tumor tissues, respectively (Figure 3). All but one non-tumor tissues and non-ß-catenin-mutated tumors demonstrated a GS and GPR49 expression ranging from 0.2 to 4 and from 0.01 to 4, respectively (Figure 3). In no tumor over-expression of only one of the two tested ß-catenin target genes was observed. In one additional adenoma (case 485), GS and GPR49 were

over expressed (39-fold and 48-fold, respectively), in the absence of detectable ß-catenin or axin1 mutation. In the group of tumors where the ß-catenin pathway was activated, including the 12 tumors mutated for ß-catenin and the case n° 485, 7 cases had a firm diagnosis of adenomas, 2 were borderline lesions between adenomas and HCC, 3 were cases of adenoma associated with an HCC and 1 was a tumor reclassified as HCC (in a young male of 15 years old) (Table 2). Male patients were over-represented in this ß-catenin activated group (5 cases, 38% p=0.02), and of the 8 females all but one female were using oral contraceptives. This group of 13 lesions presented specific characteristics when compared with non-ß-catenin activated tumors. ß-catenin activated tumors frequently showed cytological abnormalities and pseudo-glandular formation since they were observed in 69% of the cases, ($p<0.002$), whereas they were less frequently steatotic ($p=10^{-4}$, Fisher) and more frequently diagnosed as borderline lesions between adenoma and HCC or associated with HCC ($p=10^{-3}$, Fisher, Table 2, Figure 2).

c- Non-mutated adenomas and correlations with phenotype

The first two categories shown above of tumors with mutation of HNF1α and activation of ß-catenin account for 58% of the tumors. In the remaining 39 tumors not having a mutation in either of these genes, we found 17 cases with focal or diffuse inflammatory infiltrates; a much higher incidence than the two HNF1α mutated and three ß-catenin activated cases (Table 2). In this group with no mutations but showing inflammatory infiltrates, the tumors had significantly more ductular reaction (p=0.008), dystrophic vessels (p=0.02), numerous vessels (p=0.01) and cytological abnormalities (p=0.001) when compared to the whole series. All together, these inflammatory tumors resemble TFNH and accordingly, 3 diagnoses of firm TFNH and 5 borderline lesions between adenoma and TFNH were classified in this group by expert pathologists. The remaining 22 tumors consisted of lesions without HNF1α mutation, ß-catenin

activation or inflammatory infiltrate. No specific clinical or morphological features were typical for this latter group.

3- Classification of hepatocellular adenomas

Our sequencing results revealed 3 groups of HA, according to the presence of either HNF1α or ß-catenin mutations, or no mutations and in this last group, a fourth subset of tumors was defined by the presence of inflammatory infiltrates. Therefore, based on the three criteria, HNF1α mutations, ß-catenin activation and the presence or the lack of inflammatory infiltrates, we identified 4 groups of hepatocellular adenomas. These three criteria enable us to unambiguously classify 91 out of the 96 tumors in the four groups of tumors (Figure 4). Only two lesions were simultaneously inflammatory and HNF1α mutated and three others were inflammatory and ß-catenin mutated and hence difficult to classify precisely.

In an alternative analysis we first took the major clinical and morphological criteria and searched for correlations with the above defined classification. Adenomatosis and multiple adenomas seemed more likely to have HNF1α mutations but the frequency was not significantly different from that of ß-catenin activation. Microscopic hemorrhages (55 cases) and clinical hemorrhagic syndrome (32 cases) were almost equally distributed among the different defined subgroups of tumors. Interestingly, in the group of HNF1α mutated tumors, there was no significant relationship between the size of the nodules and the presence of hemorrhages (mean 85 vs 77 mm) in contrast to that observed in the non-HNF1α mutated tumors in which hemorrhages were significantly associated with higher diameter of the nodules (86 vs 53 mm, $p<0.01$, t-test). Interestingly the two cases associated with hepatic polycystic disease were HNF1α mutated whereas among the two cases of adenomatosis associated with glycogenosis type I, one was ß-

catenin mutated, when the other was non-mutated and non-inflammatory. Apart from morphological characteristics used for diagnosis of malignant tumors, HCC and borderline lesions between adenomas and HCC were significantly associated with ß-catenin activation and the absence of inflammatory infiltrates in the lesion ($p=0.004$, Figure 4).

Discussion

Our study demonstrates close genotype-phenotype correlations in hepatocellular adenomas. Our comprehensive analyses of a large series of tumors including molecular, clinical and morphological data revealed that HNF1α mutations and ß-catenin activation lead to different tumor subtypes with specific characteristics. HNF1α mutations result in adenomas that are noticeably steatotic, whereas ß-catenin activated lesions were frequently characterized by pseudo-glandular formation and cytological abnormalities. Furthermore tumors that are not mutated in either gene but that have inflammatory infiltrates seem to define a third subgroup of tumors that we termed "inflammatory adenoma". These lesions also had marked vessel dystrophy and resembled TFNH(10, 12). These results reveal a heterogeneity of HA and enable us to propose a new classification of these tumors, with some clinical implications.

Since non-inflammatory and more specifically ß-catenin activated HA are most frequently associated with HCC or borderline tumors where the differential diagnosis between adenoma and HCC is difficult, we can hypothesize that these tumors have a higher risk of malignant transformation. Consequently, patients falling into this subgroup could be closely followed to detect any early recurrence.

Mutations of HNF1α and activation of the ß-catenin pathway are found in benign hepatocellular tumors, suggesting these genes play a role at an early stage of hepatocellular tumorigenesis. In three patients, we identified the same nucleotide mutation of ß-catenin in the adenoma and in the HCC part of the tumors (data not shown) indicating that the benign and malignant lesions had the same clonal origin. Since HCC or borderline tumors are more frequently associated with ß-catenin activation, alteration of this pathway may promote a faster malignant transformation than HNF1α inactivation. These results are corroborated by the high frequency of ß-catenin activating

mutations found in HCC (20 to 34% of the cases(21-24)) contrasting with the low frequency of HNF1α inactivation in HCC (3 mutated cases out of 120 screened HCC (5) and unpublished data).

Inflammatory adenomas not carrying mutations in either of the above two genes shared morphological and genetic features with TFNH for which no HNF1α or ß-catenin mutation was observed(12). This result permits us to propose TFNH as part of a larger group of "inflammatory adenomas". As with the ß-catenin mutated lesions, the inflammatory adenomas were frequently associated with cytological abnormalities, but considered more probably dystrophic than premalignant, since no cases of borderline lesions between adenoma and HCC or adenomas associated with HCC were observed in this inflammatory adenoma group. These results should be confirmed in a prospective study in order to translate them in clinical guidelines, since the diagnosis of liver adenoma remains a challenge even for liver pathologists. This is well illustrated in this retrospective study regarding the diagnosis of benign hepatocytic nodules proposed by each pathologist. Difficulties leading to the lack of diagnostic consensus were mainly for nodules looking-like adenomas but with some features of FNH classified as adenomas by some, and TFNH by others and for nodules with cytological abnormalities classified as adenomas with areas of HCC or borderline lesions. This genotype/phenotype correlations might facilitate classification of these "borderline" tumors.

An accurate adenoma classification is also important to elucidate genetic predisposition to develop hepatocellular adenomas. We previously showed that germline HNF1α mutations predispose to liver adenomatosis and maturity onset diabetes type 3 (MODY3)(5-7). These observations suggested that relatives of patients presenting germline HNF1α mutation should be investigated for familial liver adenomatosis. Other genetic predispositions may be identified,

particularly to explain the occurrence of multiple adenomas in patients. We showed by genotyping different nodules in the same patients that multiple lesions may have different HNF1α mutations, indicating their independent origin but simultaneous development (data not shown). Furthermore, the incomplete penetrance of the adenomatosis phenotype in HNF1α germline mutated patient may suggest the existence of modifier genes. Interestingly, our familial adenomatous polyposis patient associated with an APC germline gene mutation also showed HNF1α somatic mutations in its hepatocellular adenoma without loss of the second APC allele and without over-expression of ß-catenin targetted genes. This observation contrast with a previously reported cases of hepatocellular adenoma related to FAP and presenting a ß-catenin activation(35, 36).

Finally, such classification will facilitate the search for specific alterations of carcinogenetic pathways by providing homogeneous subgroups of tumors for comparison or study. The molecular and pathological classification of hepatocellular adenomas permits the identification of strong genotype-phenotype correlations and suggests that adenomas with ß-catenin activation have a higher risk of malignant transformation. Furthermore, a genetic counselling should be recommended to patient with adenomatosis in particular when steatotic or with familial history to search for germline HNF1α mutation.

Table 1: HNF1α mutations identified

N°ID	Tumor Tissue		Non tumor tissue
	Allele 1	Allele 2	
340*	685C>T, R229X	LOH	R229X
357*	IVS2+1G>A	618G>T, W206C	nm
358*	617G>T, W206L	872_884del, P291fs	nm
368*	710A>G, N237S	LOH	nm
369*	436_437delC, Q146fs	LOH	nm
370*	872_873insC, P291fs	803T>G, F268C	nm
371*	617G>T, W206L	730A>G, R244G	nm
373*	82C>T, Q28X	LOH	nm
380	196G>T, E66X	779C>T, T260M	nm
383	493T>A, W165R	1340C>T, P447L	nm
385	817A>G, K273E	LOH	nm
461	872_873insC, P291fs	872_873delC, P291fs	nm
462	632A>C, Q211P	617G>T, W206L	nm
464	71_82del, A25_Q28del	747_764del, Q250_G255del	nm
474	617G>T, W206L	LOH	nm
476	232_245dup, T81fs	1288_1289delG, G430fs	nm
479	476_479del, R159fs	811C>T, R271W	nd
482	653 A>G, Y218C	LOH	nd
487	814 C>A, R272S	LOH	R272S
489	IVS1-2 A>T	1072_1073delCins11, P358fs	nd
508	77T>A, L26Q	872_873delC, P291fs	nm
509	391C>T, R131W	872_873delC, P291fs	P291fs
514**	829_837del, F277_H279del	872_873insC, P291fs	P291fs
516	185_194del, N62fs	788G>A, R263H	nm
518#	164_168del, G55fs	LOH	G55fs
523**	872_873insC, P291fs	LOH	P291fs
532	198_202del, T67fs	618G>T, W206C	nm
535	617G>T, W206L	872_873insC, P291fs	nm
539	686G>A, R229Q	775G>C, V259L	nm
540	618G>T, W206C	LOH	nm
546	1 A>G, M1X	620G>A, G207D	nm
575	IVS2+1 del13	956_957delG, G319fs	nm
583	811_818del, R271fs	815G>A, R272H	nm
584	607C>T, R203C	710A>G, N237S	nm
590	257_258del, L86fs	IVS2+1 G>T	IVS2 +1 G>T

591	787C>T, R263S	LOH	nm
592	526C>T, Q176X	IVS5-2 A>G, S371fs	nm
635	618G>C, W206C	872_873insC, P291fs	nm
682	197_198insA, T67fs	872_873insC, P291fs	nm
687	814C>A, R272S	LOH	nm
694	682G>T, E228X	IVS2_2 A>G	nm
696	872_873insC, P291fs	1168G>T, E390X	nm
699	685C>G, R229G	710_711insA, N237fs	nm
705	618G>T, W206C	631C>T, Q211X	nm

fs: frameshift; ins:insertion; del:deletion; nm: non-mutated; previously described in *Bluteau et al.(5), in **Reznik et al.(7) and in #Bacq et al.(6)

Table 2: Main clinical and pathological characteristics according to the genotype classification

	HNF1α mutated n=44	ß-catenin activated n=13	Non-mutated n=39	P value*	Non mutated cases	
					Inflamatory n=17	Non-inflamatory n=22
Mean age ± SD (y)	37±12	32±16	39±12	ns	42±10	37±12
Male	9% (4)	38% (5)	10% (4)	0.02	12% (2)	9% (2)
Oral contraception	67% (24)	87% (7)	73% (22)	ns	92% (11)	61% (11)
Acute Symptoms at diagnosis	25% (10)	25% (3)	35% (12)	ns	29% (4)	40% (8)
Number of tumors • Unique • 2 to 10 • >10	 43% (19) 34% (15) 23% (10)	 62% (8) 15% (2) 23% (3)	 62%(24) 25%(10) 12%(5)	ns	 65% (11) 23% (4) 12% (2)	 59% (13) 27% (6) 14% (3)
Diameter of the main lesion: mean ± SD (mm)	81±57	78±48	74±40	ns	77±39	73±42
Steatosis : • No or <10% • 10-30% • 30%-60% • >60%	 7% (3) 30% (13) 27% (12) 36% (16)	 77% (10) 0% 0% 23% (3)	 44% (17) 20.5% (8) 20.5% (8) 15% (6)	$<10^{-4}$	 47% (8) 23.5% (4) 23.5% (4) 6% (1)	 41% (9) 18%(4) 18% (4) 23% (5)
Microscopic hemorrhage	45% (20)	69% (9)	66% (26)	ns	65% (11)	68% (15)
Dystrophic vessels	23% (10)	38% (5)	46% (18)	ns	59% (10)	36% (8)
Fibrosis	36% (16)	62% (8)	36% (14)	ns	29% (5)	42% (9)
Inflammatory infiltrate	5% (2)	23% (3)	44% (17)	$<10^{-4}$	100% (17)	0
Ductular reaction	9% (4)	0%	18% (7)	ns	29% (5)	9% (2)
Cytological abnormalities	2% (1)	69% (9)	31% (12)	$<10^{-6}$	53% (9)	14% (3)
Pseudo-glandular formation	20% (9)	69% (9)	5% (2)	$<10^{-5}$	6% (1)	5% (1)
Pathological non-tumor liver (including steatosis)	16% (7)	54% (7)	58% (22)	10^{-4}	59% (10)	57% (12)
Final diagnosis: • HA • HCC • HA with HCC • TFNH • HA/HCC** • HA/TFNH** • HA/FNH**	 84% (37) 0 2% (1) 0 5% (2) 0 9% (4) 0	 54% (7) 8% (1) 23% (3) 0 15% (2) 0 0 0	 63% (24) 0 3% (1) 8% (3) 5% (2) 16% (6) 5% (2) 3% (1)	0.001	 50% (8) 0 0 18% (3) 0 31% (5) 0 6%(1)	 73% (16) 0 5% (1) 0 9% (2) 5% (1) 9% (2) 0

• Unclassified						

( ): number of cases; HA: hepatocellular adenoma; HCC: hepatocellular carcinoma; FNH: focal nodular hyperplasia; TFNH: telangiectatic focal nodular hyperplasia. *P value obtained by comparing the three groups HNF1α mutated, ß-catenin activated and non-mutated cases. **borderline lesion

Table 3: ß-catenin mutations identified in tumors.

N°ID	Nucleotide change	Amino acid change
372	101G>A	G34E
376	98C>G	S33Y
452	IVS2+147_IVS3+62del	A5_A80del
469	100G>A	G34R
517	133T>C	S45P
700	134C>T	S45F
361	7_378del	T3_A126del
477	13_241del	A5_A80del
534	55_425del,insGGT	K19_Y142del,insV
543	60_446del	A20_L149del
505	104A>T	K335I
678	IVS2-49_426del	A5_Y142del

Figure 1: spectrum of HNF1α mutations. Each bar indicates a point mutation leading to a frameshift or a stop codon (upper panel) or to an amino acid substitution (lower panel). In-frame deletion and mutations in splicing sites are indicated by a triangle and a circle, respectively. Somatic mutations are in black whereas germline mutations are in red.

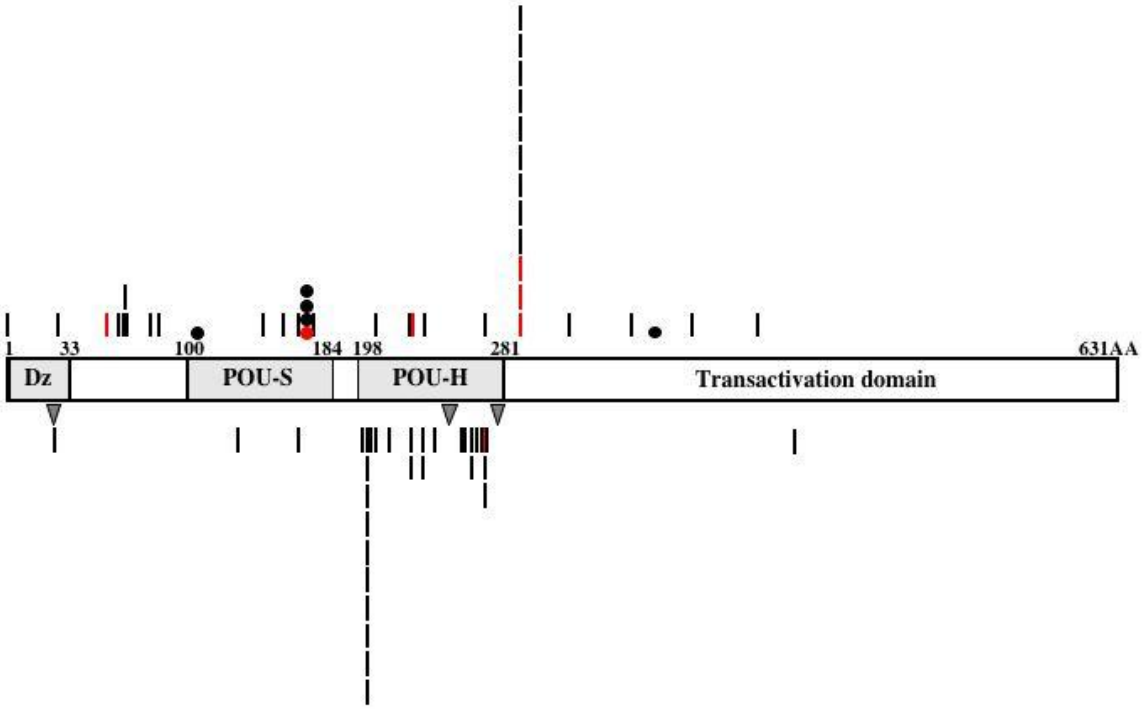

Figure 2: main characteristic morphological features in adenomas. a: typical aspect of a HNF1α mutated adenoma with marked steatosis, no cytological abnormalities and no inflammatory infiltrate (adenomatosis in a 42 year old female patient). b: a ß-catenin activated adenoma presenting pseudo-glandular formation (white arrow) and some cytological abnormalities with hyperchromatic nuclei (black arrow) in a 14 year old male patient (androgen therapy). c: an adenoma without HNF1α nor ß-catenin mutations presenting focal inflammatory infiltrate (white arrow) and some cytological abnormalities with hyperchromatic nuclei (black arrow) in a 53 year old male patient. d: an adenoma without HNF1α nor ß-catenin mutations and without any morphological particularities: no steatosis, no cytological abnormalities and no inflammatory infiltrate in a 27 year old female patient.

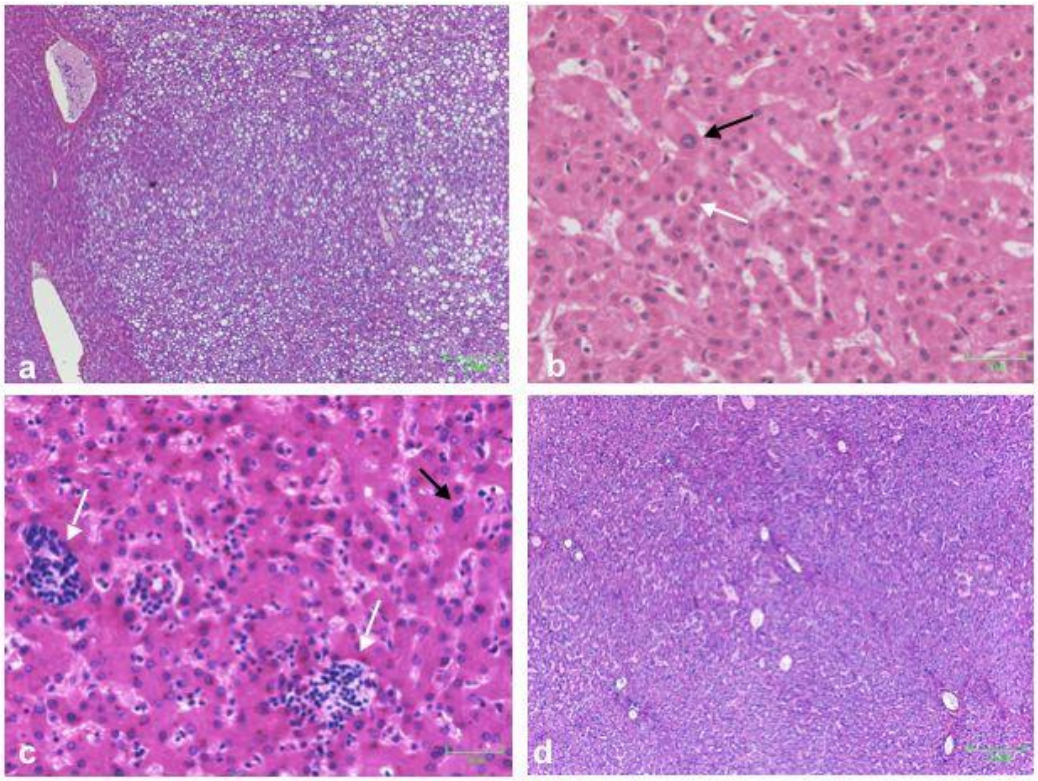

Figure 3: Activation of the ß-catenin pathway in hepatocellular adenomas mRNA expression of ß-catenin target genes glutamine synthetase (GS) and GPR49 in tumors. Mean level of expression are represented by an horizontal line. Quantitative RT-PCR analysis was performed in samples with a good-quality RNA, i.e. 71% of all samples (3 samples with a ß-catenin mutations had no qualified RNA).

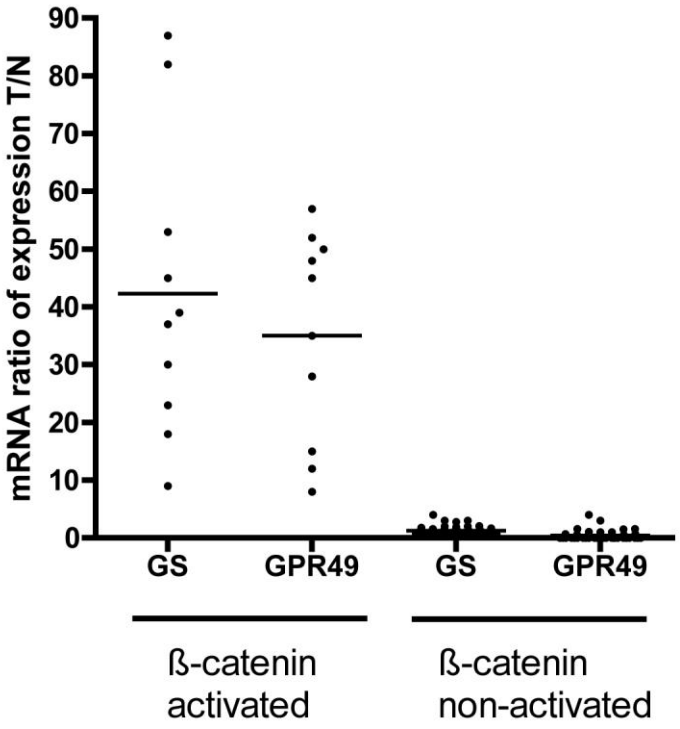

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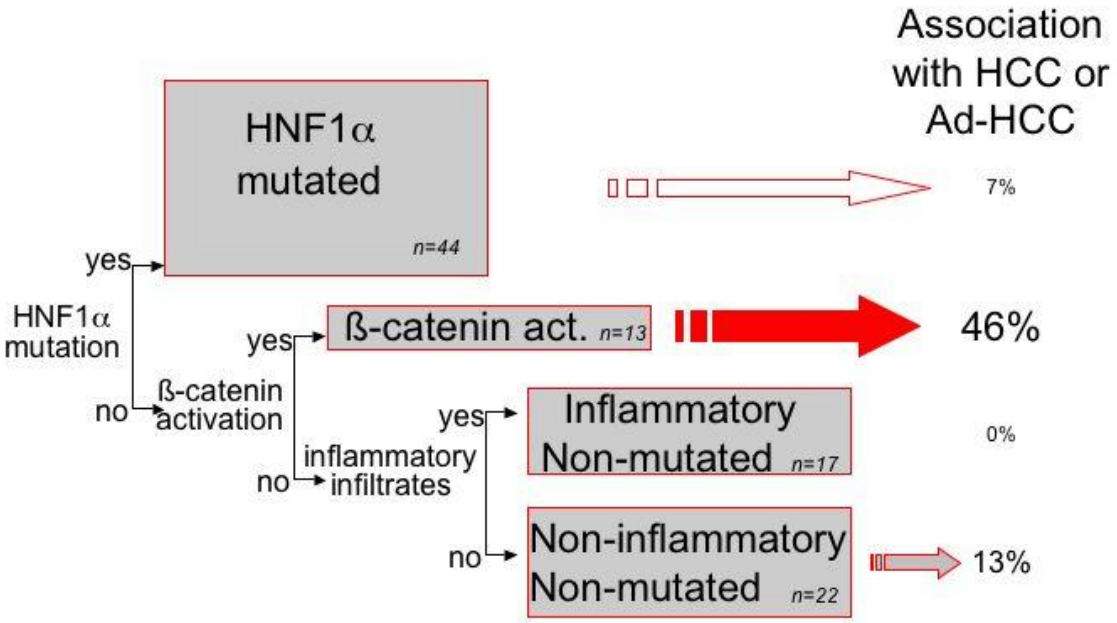
Figure 4: Schematic representation of hepatocellular adenoma classification. Frequencies of adenoma associated with HCC or of borderline lesion between adenoma and HCC (Ad-HCC) are indicated at the right for each group of tumors.



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